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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CYCLIC PEPTIDES AND THEIR USE

(57) Abstract

The present invention deals with a novel class of cyclic peptides with a selective IgG-binding activity and an inhibitory effect on the classical activation pathway of complement. These peptides may be pharmaceutically applied in compositions with an anti-inflammatory potential and further be used to enrich IgG from blood serum or plasma, to deplete plasma or serum from IgG, and/or to quantitate IgG levels in e.g. body fluids.

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Cyclic peptides and their use.

The present invention with a novel class of peptides and their application.

It is known that simple oligopeptides may display diverse and potent biological activities including antibiotic, antitumor, antiviral as well as immunosuppressive activities. Thus far, cyclic peptides were mostly of microbial and more in particular of fungus origin. These so-called cyclosporins are known as immunosuppressive compounds and are used to prevent graft rejection after organ transplantation. Disadvantages of cyclosporins are their insolubility in water and their toxicity, particularly for the kidneys.

Active peptides originating from higher plants are very rare. Recently, cyclic oligopeptides were isolated from the roots of Rubia cordifolia and R. akane (Rubiaceae). The cyclic hexapeptide was reported to possess antitumor activity in a mouse leukemia model (Itokawa, H., Takeya, Koichi, Mori, N., Kikodoro, S., and Yamamoto, H. (1984a) Studies on antitumour cyclic hexapeptides RA obtained from Rubiae radix, Rubiaceae (IV): Quantitative determination of RA-VII and RA-V in commercial Rubia radix and collected plants. Planta Med. 51, 313-316 & Itokawa, H., Takeya, K., Mori, N., Hamanaka, T., Sonobe T., and Mihara, K. (1984b) Isolation and antitumour activity of cyclic hexapeptides isolated from Rubiae Radix. Chem. Pharm. Bull. 32 284-290). Both cyclosporins and the Rubia peptides are for the major part composed of non-proteinogenic amino acids.

Proteins and perhaps also peptides may bind to the Fc-portion of immunoglobulins. A surface protein from Staphylococcus aureus (protein A) was shown to bind IgG and to enhance complement activation via the classical pathway (CP) (Masuda, S., Sakurai, S. & Kondo, I. (1975) Simple and effective method for selecting

protein A deficient mutants by cosedimentation with sensitized sheep erythrocytes. Infection and Immunity 12, 245-251; Van Dijk, H. & Van Bohemen, C.G. (1978) Indirect plaque-forming cells detected by use of normal mouse serum I. Normal mouse serum plaque-forming cells are IgA-producers. Cellular Immunlogy 38, 124-130). Protein A and an analogue isolated from Streptococcus strain G148 (protein G) are used to isolate IgG from serum and plasma (Björck, L & Kronvall, G. (1984) Purification and some properties of Streptococcal protein G, a novel IgG-biunding reagent. J. Immunol. 133, 969-973).

Leupeptin (a tripeptide from actinomycete fermentation) and single amino acids can interfere with complement activation via the CP and/or the alternative pathway (AP) (Takada, Y., Arimoto, Y. Mineda, H. & Takada, A. (1978) Inhibition of the classical and alternative pathways by amino acids and their derivatives. Immunology 34, 509-515).

It has now been found by us that there are cyclic peptides with IgG-binding properties. This is to say that cyclic peptides which are isolated from e.g. the latex of specific plants or which may be prepared synthetically or semi-synthetically, were find to bind to human but also to rabbit and mouse IgG but not to IgM and IgA in in vitro systems for IgG-binding. The peptides were isolated and identified on the basis of their selective inhibition of complement activation via the CP (Kosasi, S., Van der Sluis, W.G., Boelens, R., 't Hart, L.A. & Labadie, R.P. (1989) Labaditin, a novel cyclic decapeptide from the latex of Jatropha multifida l. (Euphorbiaceae) FEBS letters 256, 91-96). AP activation was not or only slightly affected by these peptides. It was shown that the anticomplementary activity of the peptides is mediated by an interference with C1q-acceptor sites on the IgG molecules they bind to. This means that the cyclic peptides combine protein A-like IgG-binding activity with leupeptin- and amino

acid-like anticomplementary behavior. Such combined activity has never been described in literature, not for proteins but neither for linear peptides and particularly not for cyclic peptides. The anti-complementary activity of the novel cyclic peptides is mechanistically different from that of the linear peptide AA 275-290, which represents a major part of the C1q-acceptor site on IgG (Prystowski, M.B., Kehoe, J.M., & Erickson, B.W. (1981) Inhibition of the classical complement pathway by synthetic peptides from the second constant comain of the heavy chain of IgG. Biochemistry 21, p. 6349-6358). The latter does not bind to IgG but prevents complement activation by competing with IgG for binding to C1. The anticomplementary activity is also different from the leupeptin-induced and amino acid-induced complement inhibition which is not based on binding to IgG.

An advantage of our novel peptides over other cyclic peptides, such as cyclosporins, is their extreme solubility in water (up to 1000 mg per ml) and their non-toxic behavior, at least in mice. They also differ from cyclosporins and the Rubia peptides in the fact that they are built up from proteinogenic (= proteinic) amino acids.

Therefore, the cyclic peptides all to the invention consist preferably of proteinic amino acids. This means that such amino acids do not have to be modified, e.g. by methyl groups. It should be noted that the known cyclosporins contain methylated or derived proteinic amino acids.

Structural analysis of the cyclic peptides of the present invention reveals that said cyclic peptides contain preferably the amino acid Trp and/or His, in particular the dipeptide groups Trp-Gly and/or His-Gly.

It is preferred tbat the cyclic peptides according to the invention contain 8-12, preferably 9-11 amino acid residues.

In general, the cyclic peptides according to the invention contain at least 6 amino acid residues which are preferably selected from the group consisting of Ala, GLy, Val, Trp, Thr, Ile, Ser, and Leu.

Two important examples of cyclic peptides according to the present invention are characterized by the sequence Ala-Gly-Val-Trp-Thr-Val-Trp-Gly-Thr-Ile (Labaditin) and Ala-Ser-Ile-Leu-Gly-Leu-Gly-Trp-Ala- (Biobollein).

Of course, the cyclic peptides according to the present invention may be prepared according to classical peptide synthesis methods. However, they may also be isolated from plant material of the Euphorbiaceae family, in particular the latex of Jatropha species.

The peptides according to the invention may be used for various purposes such as for the preparation of pharmaceutical compositions or for analysis, and/or separation standardization purposes.

The use of the cyclic peptides according to the invention may - in general - be used for IgG-binding and anticomplementary activity in mammals including human beings.

The present invention also relates to the application mentioned above. The present invention further relates to a method for treating diseases such as inflammatory diseases including rheumaticas well as other systemic or local auto-immune, and immune complex-related diseases including extrinsic allergic alveolitis in mammals including human beings wherein a cyclic peptide as defined in the above is used as an active substance.

The present invention further relates to pharmaceutical compositions for treating diseases such as inflammatory diseases including rheumatic as well as other systemic or local auto-immune, and immune complex-related diseases including extrinsic allergic alveolitis, said compositions containing a cyclic peptide as

defined in the above.

In general, the peptides according to the invention may be applied in composition with an anti-inflammatory potential and further be used to enrich IgG from blood serum or plasma to deplete plasma or serum from IgG, and/or to quantitate IgG levels in e.g. body liquids.

With respect to the anticomplementary activity the following is remarked.

Complement is an important system in the body's defense against foreign invaders such as bacteria, virusses, and other micro-organisms. The activation of the complement cascade by foreign materials leads to inflammation, opsonisation by C3b for phagocytosis, and the lysis of cells by membrane damage. Complement can also be activated in diseases such as immune complex and/or auto-immune diseases and immunity states where tissue damage may occur. It is believed that inhibition of the complement cascade can prevent tissue injury. A brief review on the CP and AP complement inhibitors is given in Ashgar, S.S., (1984) Pharmalogical Manipulation of Complement System, Pharmalogical Reviews 36, 223-224. Up to now, a limited number of anticomplementory agents are available. Of these agents only cobra venum factor (CVF) gives rise to efficient complement-depletion in vivo. The complement-depletion brought about by CVF, however, is not selective but involves both the CP and the AP complement activation. Therefore, the new C-inhibitors according to the present invention for the treatment of auto-immune and other immune complex diseases are very important. The cyclic peptides according to the invention have specificity for the CP and leave the AP unaffected. The latter is essential not only for the host's general defense potential but also for the elimination of certain types of immune complexes (Vogt, W., (1985) Drugs and the complement system. Trends in Pharmocol. Sciences 6, 114-119). Probably, the

best CP-inhibitors are substances which interfere with the binding of C1 to immune aggregates.

Since the cyclic peptides according to the invention cause an inhibition of the classical complement pathway and leave the AP functionally intact, it may be assumed that the peptides will not interfere with the non-specific defense of the body against microbial infections and with processes as the AP-dependent elimination of immune complexes from the circulation. This means that the cyclic peptides according to the invention are highly interesting substances that are suited to treat the deleterious effects of the CP-activation in vivo as occurring in auto-immune diseases.

It is a very important feature of the substances of the invention that no acute toxic effects can be shown in mice in concentrations up to 5 mg per animal. Peptides according to the invention could be beneficial not only by local application (e.g. in vasculitis) but may also be of use upon oral or parenteral administration in the case of diseases such as mentioned above and in arthritis, hepatitis, glomerulo-nephritis etc. It is expected that the cyclic peptides according to the present invention will not show chronic toxicity, either.

The cyclic peptides may be used in the estimation of complement-activating human IgG's and analogues in other species by ELISA, the isolation of these antibodies and analogues by affinity chromatography, very similar to protein A-sepharose chromatography, and the selective removal of IgG from the circulating blood in immune complex diseases and cases of M. Kahler. This could probably be achieved by plasmapheresis and passing the plasma over micro-carriers (beads) coated with cyclic peptides according to the invention, e.g. labatidin or biobollein.

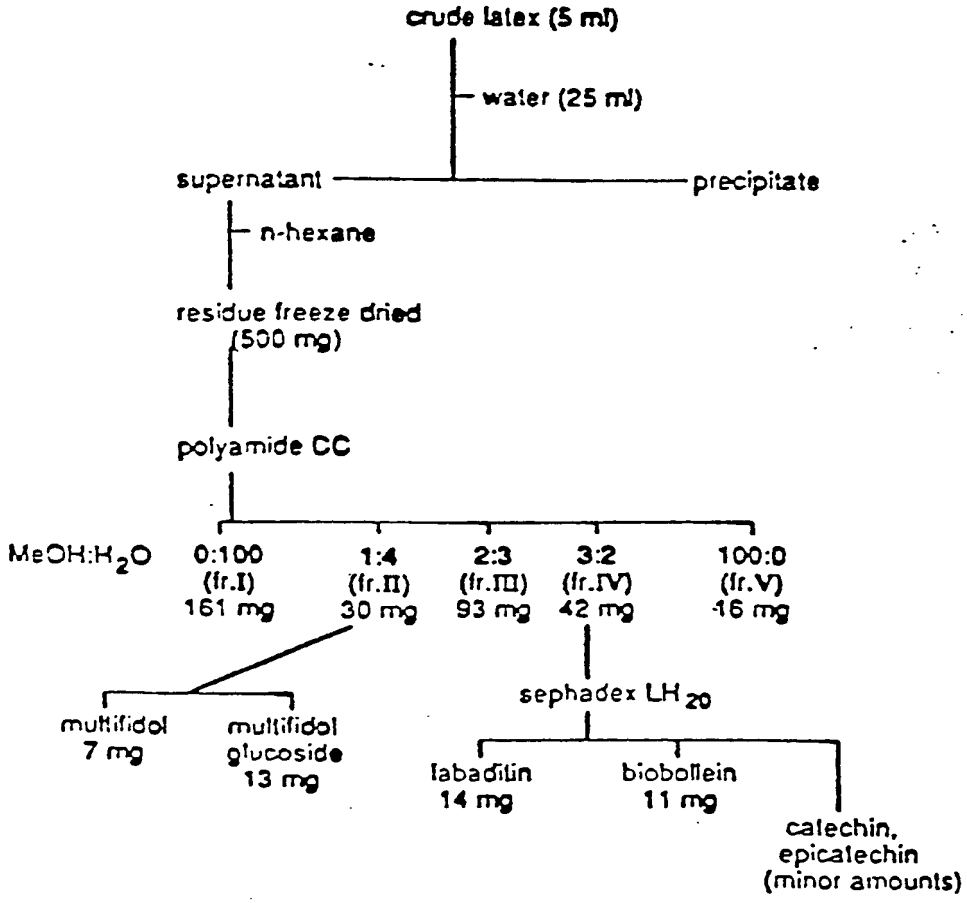
The cyclic peptides according to the invention may be prepared according to standard procedures for the synthesis of cyclic (oligo) peptides. These procedures are well known to the man in art.

However, as noted above, the cyclic peptides according to the invention may be isolated from plant material, e.g. of the Euphorbiaceae family, in particular the latex of the genus Jatropha, e.g. Jatropha multifida L.

The isolation of the cyclic peptides according to the invention is based on their modulatory effects on specific immunological parameters in vitro. Relevant experiments are carried out according to standard procedures.

Below an example is given of the isolation of two important cyclic peptides according to the invention, i.e. labaditin and biobollein.

Immunomodulatory constituents were isolated from the latex of Jatropha multifida according to the following fractonation scheme:



LAST ADDED SHEET

According to the above scheme the crude latex (5 ml) was mixed with 25 ml of demineralized water. After extraction of the supernatant with n-hexane, the supernatant was lyophillized, yielding a solid (500 mg). The solid material was dissolved in a small amount of water and subsequently separated on a polyamide column. By elution with 500 ml portions of different methanol-water mixtures, i.e. (0 : 100), (1 : 4), (2 : 3), (3 : 2) and (100 : 0) successively, five fractions (I-V) were obtained. These fractions were tested for modulatory effects on both CP and AP activation of human complement, and on the production of reactive oxygen species (ROS) by zymosan-stimulated human polymorphonuclear neutrophilis (PMN) monitored as chemiluminescence. Fraction IV was found to possess significant activity.

From fraction IV a novel cyclic decapeptide (labaditin) and a novel cyclic nonapeptide (biobollein) were isolated, both of which show a strong inhibitory effect on CP acitivity of human complement. The isolation of labaditin and biobollein respectively is elucidated here below.

Isolation of labaditin

The concentrated MeOH : H_2O (3 : 2) fraction (fraction IV) (42 mg) was dissolved in 1% $NaHCO_3$. The solution was exhaustively with ethyl acetate. Ethyl acetate extracts were combined and the solvent was evaporized under reduced pressure. The residue was dissolved in 2 ml of MeOH and separated by gel permeation over Sephadex LH-20 (40 cm x 1 cm i.d.) with MeOH as eluting agent. Per fraction 300 drops were collected. Fractions 4, 5 and 6 showing one single spot on TLC were combined. The MeOH was evaporated under reduced pressure. Subsequently, water was added and the solution was lyophillized, yielding 14 mg of a white solid.

Isolation of biobollein

The MeOH : H_2O (3 : 2) fraction (fraction IV) (42 mg) was directly extracted with ethyl acetate. The ethyl acetate extracts were combined and the solvent was evaporated under reduced pressure. The residue was dissolved in 2 ml of MeOH and by adding 15 ml of acetone : water (1 : 1), a precipitate was obtained. The precipitate was dissolved in 2 ml of MeOH and separated over Sephadex LH-20 (column 40 cm x 1 cm i.d.) with MeOH as eluting agent. Per fraction 300 drops were collected. Fractions 4, 5 and 6 showing two spots of TLC were combined and the solvent was removed under reduced pressure. The fraction constituents were separated by preparative TLC on silica gel 60 F 254, 1 mm (Merck, Darmstadt, FRG) with $CHCl_3$: MeOH : H_2O (13 : 10 : 2) as eluent (saturated chamber), and were detected under UV 254 nm. The procedure yielded two white solid compounds, i.e. labaditin (14 mg) and biobollein (11 mg).

The structure of labaditin and biobollein was determined by means of the following procedures:

Amino acid analysis

The amino acid composition was determined with an automatic amino acid analyser (LKB 4151 Alpha plus, Na-system 20 cm column) after hydrolysis in 6 N HCL at 110°C for 48 hours and, for tryptophan determination, in 6N HCL with 4% thioglycolic acid at 110°C for 24 hours.

Thin layer chromatography (TLC)

Silica gel 60 F-254 TLC plates (Merck, Darmstadt, FRG) were used with $CHCL^3$: MeOH : H_2O = 13 : 10 : 2 as solvent system (saturated chamber). Spots were visualized under UV 254 nm and by spraying with vanilin-sulphuric acid followed by heating at 110°C for 5 min.

NMR spectroscopy

For NMR experiments the purified peptide was dissolved in DMSO-d6 (conc. 30 mg/ml). For some experiments 5% D_2O was added to exchange amide protons. ^{1}H-NMR spectra were recorded on a Bruker WM-300 spectrometer at 303 and 338 K. Two-dimensional ^{1}H-NMR spectra were obtained at 303 K. For the COSY spectrum 257

records of 2K data were recorded at 400 MHz on a Bruker MSL-400 apparatus. The phase sensitive NOESY experiment containing 350 records of 2K date was obtained at 600 MHz on a Bruker AM-600. The NOESY data were multiplied with sinebell windows and Fourier transformed in both domains. The COSY spectrum was displayed in the absolute value mode.

FAB-MS measurement

For the FAB experiments a ZAB-2F VG instrument was used.

C l a i m s

1. Cyclic peptides having Ig-binding properties.

2. Cyclic peptides according to claim 1, characterized by their anti-complementary activity.

3. Cyclic peptides according to claim 1 and 2, characterized by their selective inhibitory effect on the classical activation pathway of the complement system.

4. Cyclic peptides according to claim 1-3, characterized by their solubility in water.

5. Cyclic peptides according to claim 1-3, characterized in that they consist of proteinic amino acid.

6. Cyclic peptides according to claim 1-3, characterized in that they contain Trp and/or histidine.

7. Cyclic peptides according to claim 6, characterized in that they contain the dipeptide group Trp-Gly.

8. Cyclic peptides according to claim 1-3, characterized in that they contain at least 6 amino acid residues.

9. Cyclic peptides according to claim 8, characterized in that they contain 8-12, preferably 9-11 amino acid residues.

10. Cyclic peptides according to claim 1-3, characterized in that they contain residues from amino acids selected from the group consisting of Ala, Gly, Val, Trp, Thr, Ile, Ser and Leu.

11. Cyclic peptide according to claim 1-3, characterized by the sequence Ala-Gly-Val-Trp-Thr-Val-Trp-Gly-Thr-Ile (Labaditin).

12. Cyclic peptide according to claim 1-3, characterized by the sequence Ala-Ser-Ile-Leu-Gly-Leu-Gly-Trp-Ala- (Biobollein).

13. Cyclic peptides according to claim 1-3, characterized in that they may be isolated from plant material of the Euphorbiaceae family, in particular plant material of the genus Jatropha.

14. Use of cyclic peptides as defined in any of claims 1-13 for the preparation of pharmaceutical compositions or for analysis, standarization and/or separation purposes.

15. Use of cyclic peptides as defined in any of claims 1-13 for IgG binding in mammals including human beings.

16. Methods for treating diseases such as inflammatory diseases including rheumatic, auto-immune and immune-complex related diseases in mammals including human beings wherein a peptide according to any of claims 1-13 is used as an active substance.

17. Pharmaceutical compositions for treating diseases such as inflammatory diseases including rheumatic as well as other systemic or local auto-immune and immune-complex related diseases including extrinsic allergic alveolitis, characterized in that they contain a cyclic peptide as defined in any of claims 1-13.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 91/00066

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 7/64, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC[5]	C 07 K, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	FEBS Letters, volume 256, no. 1-2, 1989, Federation of European Biochemical Societies, (Amsterdam, NL), S. Kosasi et al.: "Labaditin, a novel cyclic decapeptide from latex of Jatropha multifida L. (Euphorbiaceae)", pages 91-96 see the whole document, especially page 91, column 2 and page 95, figures cited in the application	1-11,13-14, 17

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* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th June 1991	27. 08. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Danielle van der Haas

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers *, because they relate to subject matter not required to be searched by this Authority, namely:

* 15 - 16
Please see RULE 39.1 (iv) -PCT
Methods for treatment of human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.